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Dysregulation of ornithine decarboxylase activity, apoptosis and Bcl-2 oncoprotein in Syrian hamster embryo cells stage-exposed to di(2-ethylhexyl)phthalate and tetradecanoylphorbol acetate

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Perturbations of cell proliferation and death are considered as essential events in the process of carcinogenesis. Thus, two parameters, ornithine decarboxylase (ODC), an enzyme closely related to cell proliferation and transformation, and apoptotic phenomenon are profoundly modified. Using Syrian hamster embryo (SHE) cells, we have examined in the framework of two-stage carcinogenesis (initiation–promotion) the effects of a non-genotoxic [diethylhexylphthalate (DEHP)] or genotoxic [benzo[*a*]pyrene (BaP)] carcinogen or a non-carcinogenic compound [phthalic anhydride (AP)] on these parameters. Immunoreactive Bcl-2 and Bcl-x_L proteins were also investigated following two-stage exposures. Whereas exposures to BaP, DEHP or AP alone did not provoke any modification of ODC activity, the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), strongly increased it. Using two-stage exposure protocol (xenobiotics first, then replacement by TPA-promoter), the ODC activity was higher than that obtained with TPA alone. This superinduction of ODC activity was observed only with the carcinogenic compounds DEHP and BaP. Following the same exposure protocol, spontaneous cellular apoptosis was decreased. Furthermore, Bcl-2 oncoprotein was also upregulated approximately 8- and 11-fold for BaP and DEHP respectively; meanwhile Bcl-x_L protein rate did not change. The non-carcinogenic compound AP slightly inhibited spontaneous SHE cell death without ODC superinduction. Exogenous polyamines, putrescine, spermidine and spermine diluted in the medium did not inhibit spontaneous apoptosis. Although inhibition of apoptosis was not specific of carcinogenic compound, both superinduction of ODC activity and inhibition of apoptosis via Bcl-2 upregulation, may cooperate during early stages of the carcinogenic process.

Introduction

Di(2-ethylhexyl)phthalate (DEHP*) is a well-known agent used in polyvinylchloride (PVC) industry for its plasticizing capacities. The compound is not covalently linked to plastic polymers and can be released in the environment when manufacturing plastic products, when they are used and after disposal (1). This pollutant was detected in lipophilic solutions, in some blood preparations preserved in plastic bags, and a case of inhibition of human platelet function by DEHP contaminant has been reported after transfusion (2,3). The most important risk related to DEHP was because of the teratogenic and carcinogenic properties of the chemical. *In vivo* long-term feeding of DEHP to Fischer 344 rats and B6C3F1 mice increased the percentage of hepatocarcinoma in both sexes (4). Surprisingly the compound did not show any mutagenic potential. This non-genotoxicity was confirmed by mutagenic studies with *Salmonella typhi* strains TA98, TA 100, TA1535, TA1537, TA102, with L5178Y mouse lymphoma cells and with *Drosophila melanogaster*. Negative results were also obtained with a rat hepatocyte micronucleus assay (5), with rat and hamster hepatocyte DNA damage studies and genotoxicity *in vivo* studies with Syrian golden hamster (6). As described for some hypolipidemic drugs that induce peroxisome proliferation, DEHP is listed in a special class of carcinogens: peroxisome proliferator tumor promoters (PPs). In the two-stage carcinogenesis model (initiation–promotion), non-genotoxic chemicals were considered for a long time to be active at the promotion stage. At present this concept does not prevail: several non-genotoxic chemicals are able to induce tumors in rodents (7). In the two-stage SHE cell transformation assay, some of these chemicals such as chlordane, a pesticide, or clofibrate, a hypolipidemic drug, were active at the initiation stage of the transformation process (8,9). In short-term and long-term rodent liver assays, controversial results were reported concerning the mode of action of DEHP. Long-term studies with female rats showed that DEHP was negative for promotional activity (10). This was confirmed in short-term assays that evaluated hepatocellular altered foci in the rat (11–13). On the other hand, these *in vivo* assays did not demonstrate any initiating activity of DEHP. Therefore it is thought that DEHP may act as a complete hepatocarcinogen.

One major feature observed in cancer cells is the dysregulation of specific enzyme systems. With this fact in mind, the role of ornithine decarboxylase (ODC, EC 4.1.1.17, 53 kDa) has been investigated. ODC is the first and rate limiting enzyme of the biosynthetic pathway of polyamines, which are polycationic biocomponents closely related to cell proliferation (14). ODC activity increased in various human and mammalian tumoral tissues (15,16). In colorectal carcinomas ODC level was higher than that in adenomas, and far higher than that observed in normal mucosa (17). Some relationship was observed between a high rate of ODC and neoplastic transformation *in vitro*. ODC activity strongly increased in Nemalite transformed SHE cell line, SHE-Tr (18); the transforming capacity of activated *ras* oncogene was enhanced in R6 fibroblasts overexpressing ODC (19); and in transfected NIH/3T3 cells the increase of ODC activity correlated with increased cell transformation rate (20–22). In transgenic mice overexpressing ODC, the frequency of spontaneous skin tumors also increased (23). In relation to oncogene expression, products of

*Abbreviations: ODC, ornithine decarboxylase; SHE, Syrian hamster embryo; DEHP, diethylhexylphthalate; BaP, benzo[*a*]pyrene; AP, phthalic anhydride; PVC, polyvinylchloride; PPs, peroxisome proliferator tumor promoters; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BrdU, bromodeoxyuridine; CAM, camptothecin; GGT, gammaglutamyl-transpeptidase; DEN, diethylnitrosamine.

c-myc and *c-fos* oncogenes have been shown to be transcriptional factors of ODC (24,25). In contrast, blocking endogenous ODC by inhibitor or by using antisense ODC–RNA prevented NIH 3T3 cell transformation induced by *src* oncogene. ODC may intervene in the signaling pathway of various oncogenes, which might intervene in increasing tyrosine phosphorylation, especially of pp130^{CAS} substrate (26). Inhibition of ODC metabolism by the drugs, alpha-difluoromethylornithine and eflornithine has been studied in cancer chemoprevention. These drugs were also used in clinical trials to improve anticancer chemotherapy in association with polyamine deprivation (27–29).

In cancer research, the dysregulation of cell proliferation has long been the major mechanistic exploration until the data reported by Kerr *et al.* (30) that described the physiological process of apoptosis. This phenomenon of active cell death was involved in embryo development and could be implicated in various human diseases such as neurodegenerative disorders, infectious diseases, heart diseases and cancer (31). Apoptosis was tightly regulated and several molecular factors intervened inside and outside the cell to control the programmed cell death pathway. Some proteins such as Myc, AP-1, transcription factors, wild-type p53 and Bax were able to induce cell death. Others such as Ras, Raf, pRb, Bcl-2 and Bcl-x_L prevented cells from undergoing apoptosis. However, the molecular regulation of cell death was far more complex. Internal rheostats such as Bcl-2/Bax or Bcl-x_L/Bcl-x_S ratios could intervene in promoting or repressing apoptosis as well (32). In cancer development, it is postulated that inhibition of apoptosis may help initiated cells to escape cell death and acquire a tumorigenic phenotype (33,34).

Within the framework of the two-stage carcinogenesis, we have studied the mode of ODC induction in stage-exposed SHE cells to the carcinogens DEHP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In comparison, the same exposure protocol was used with genotoxic carcinogen benzo[*a*]pyrene (BaP) and with phthalic anhydride (AP), a non-carcinogenic derivative. The results show that ODC superinduction is regularly obtained after stage exposure of SHE cells to carcinogens. This correlates with inhibition of apoptotic rate and upregulation of Bcl-2 oncoprotein in these cells.

Materials and methods

Chemicals and reagents

Di(2-ethylhexyl)phthalate, phthalic anhydride, BaP and polyamines (putrescine, spermidine, spermine) were purchased from Sigma (France), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) from LC Services Co, Woburn (MA) and L-[1-^{14}C]ornithine from Amersham (UK). Cell culture medium (DMEM) from Gibco BRL-Europe (France) and fetal calf serum from Hyclone Europe (Belgium). Polyclonal anti-Bcl-2 antibody (Ab2 clone) and anti-Bcl-x_L antibody from Calbiochem (USA) and anti-rabbit IgG peroxidase conjugate from Sigma (France). Other chemicals are of RP grade.

Cell cultures and treatments

In this work, we used primary cultures of Syrian hamster embryo (SHE) cells. These are representative of normal diploid cells, genetically stable, have a finite lifespan in culture and are capable of activating a wide spectrum of chemical carcinogens. These cells are extensively used in SHE cell morphological transformation assays and are a useful method for detecting the carcinogenic potential of chemicals (35,36). Its sensitivity was highly increased by the adoption of a two-stage protocol (initiation–promotion) (37). Primary cell cultures were prepared from 13-day-old golden Syrian hamster embryos, as described by Pienta *et al.* (38) modified by Elias *et al.* (39). In preliminary experiments, the first cell batch was tested for its capacity to develop transformed colonies in the presence of 4 μM of BaP in a morphological transformation assay. Positive cell batches were stored in liquid nitrogen. Only early diploid cell passages were used in the present study.

For the ODC activity assay, the cells (2×10^4) were seeded into 12-well culture plates in DMEM medium, pH 7.4, supplemented with 15% fetal calf serum. At 72 h later they were submitted to chemical treatments as previously described (40). Different treatment protocols were used to determine optimal time of exposure and optimal doses of each chemical. In the two-stage protocols we explored (i) the modulatory effect of DEHP, AP and BaP on TPA-induced ODC activity, and (ii) changes of ODC activity after cell pre-exposure to these chemicals (initiation stage) and to TPA. The same treatment protocols were used in the studies of the effects of chemicals on cellular apoptosis.

Assay of ODC activity

ODC activity was determined in cell cultures by measuring the $^{14}CO_2$ released from ^{14}C-labeled L-ornithine as described (40). Briefly, lysed cell cultures were incubated at 37°C, in 50 mM Tris–HCl buffer, pH 7.5, 40 mM pyridoxal phosphate, 2.5 mM dithiothreitol, 0.4 mM L-ornithine [containing ^{14}C-labeled L-ornithine at 0.1 μCi (3.7 kBq)] to make a 0.4-ml final volume. After 1 h of incubation the enzymatic reaction was stopped by injecting 50 μl 2 N perchloric acid and the incubation was continued for another hour. The ^{14}C-labeled CO_2 released was entirely adsorbed onto a filter disc (Whatman GF/C) and the radioactivity determined by liquid scintillation counting (Beckman counter LS-6000-TA). Protein concentration was measured according to Bradford's microassay (41) using a Labsystems Multiskan MS microplate reader. Statistical analyses were performed using Student's *t*-test.

Determination of apoptosis

The basal apoptosis was determined by quantification of the cellular DNA fragmentation using the Boehringer Mannheim kit. This assay is based on the quantitative sandwich enzyme immunoassay principle using two mouse monoclonal antibodies directed against DNA and bromodeoxyuridine (BrdU), which allows the specific detection and quantification of BrdU-labeled DNA-fragments.

The assay was processed as described by the manufacturer. Briefly, SHE cells were labeled with 10 μM BrdU overnight. Then, they were collected, seeded at 5×10^4 cells per well and treated with chemicals. After treatment, the cells were lysed, centrifuged and an aliquot of the supernatant containing apoptotic DNA fragments was transferred to an anti-DNA (MCA-33 clone) pre-coated microtiter plate for one night at 4°C. After washing, the immunocomplexed BrdU-labeled DNA-fragments were denatured and fixed on the surface of the microtiter plate by microwave irradiation. In the next step, anti-BrdU peroxidase conjugate (BMG 6H8 clone) was allowed to react with BrdU incorporated into DNA. The amount of peroxidase was determined photometrically with TMB substrate at 450 nm with background subtraction at 690 nm. Statistical analyses were performed using Student's *t*-test.

In order to corroborate the presence or absence of apoptotic cells, each well was carefully screened by phase contrast microscopy before apoptotic measure.

Immunoblotting

Treated SHE cells (4×10^6) were scraped in ice-cold lysis buffer (containing 50 mM Tris, pH 8.0, 200 mM NaCl, 0.1% SDS, 1% Triton X100, 1 mM EDTA and 0.2 mM phenylmethyl-sulfonylfluoride). After 30 min of ice-cold incubation, insoluble materials were removed by 15 min of centrifugation at 10 000 r.p.m. Protein concentrations were measured using Biorad DC protein assay and 60 μg proteins per sample were subjected to 15% SDS-polyacrylamide gel electrophoresis. The proteins separated in the gel were transferred to a polyvinylidene difluoride membrane (Polyscreen PVDF transfer membrane, Dupont NEN, USA) by electroblotting (15 V, +4°C, overnight) (42). All the following steps were performed at room temperature. The PVDF membrane was firstly washed in Tris buffered saline containing 0.05% Tween 20 (TTBS, 20 mM Tris–HCl, pH 7.5, 500 mM NaCl and 0.05% Tween 20). In order to remove SDS, it was blocked with 5% dry skimmed milk in TTBS for 2 h. After washing, the membrane was overlaid for 1 h with the first anti-Bcl-2 (Calbiochem, Ab-2 clone) antibody diluted in TTBS. Then the membrane was washed three times with TTBS and incubated for 1 h with the secondary antibody (antirabbit IgG peroxidase conjugate). Visualization of second antibody was performed according to the Renaissance chemiluminescence protocol (Dupont NE). Previous experiments with positive control MCF7 cells, as indicated by Calbiochem, permitted a localization of the Bcl-oncoprotein band. Owing to Dupont stripping membrane protocol, the same membrane can be probed with different antibodies. Similar experiments were performed with anti-Bcl-x_L antibody (Calbiochem, Ab-1 clone).

Results

Effects of chemicals on ODC activity

Preliminary experiments permitted the sequential order as well as the optimal time and doses of treatment to be determine

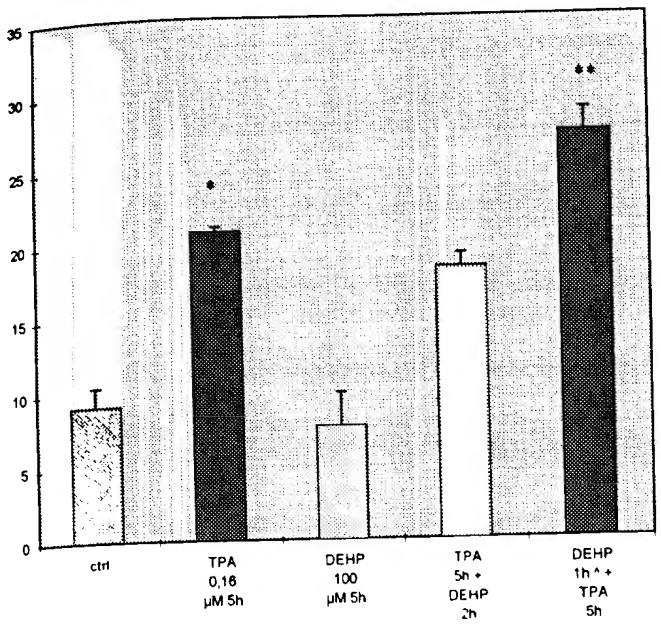

Fig. 1. Superinduction of ODC activity in SHE cells stage-exposed to di(2-ethylhexyl)phthalate and TPA. The four first treatments consisted of 1 h with treatment medium alone (DMEM medium pH 6.7 supplemented with 5% fetal calf serum) then removal and replacement for 5 h with medium, 0.16 µM TPA, 100 µM DEHP or co-treatment (TPA + DEHP) respectively. For the fifth treatment of DEHP 1 h + TPA 5 h = pre-treatment with 100 µM DEHP for 1 h then removal and replacement for 5 h with TPA. *Result significantly different from control ($P < 0.05$), **ODC stimulation significantly different from that obtained with TPA alone ($P < 0.05$). Results are of triplicate determinations and a second experiment yielded similar results.

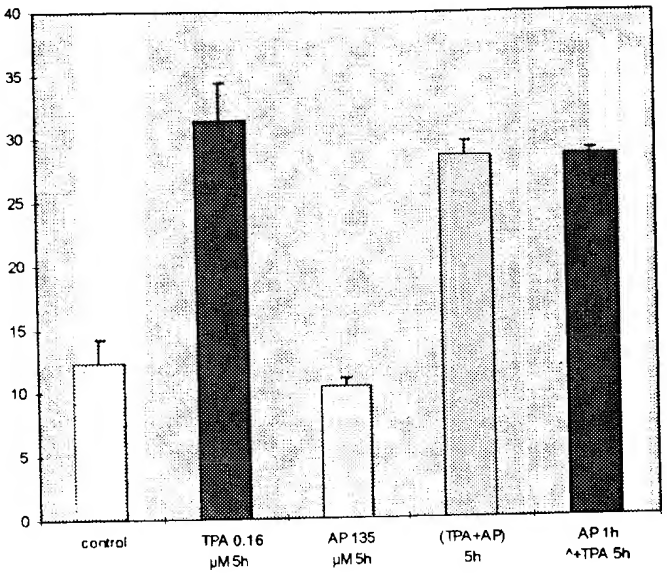

Fig. 2. ODC activities in SHE cells after different phthalic anhydride and TPA treatments. The four first treatments consisted of 1 h of treatment medium alone (DMEM medium pH 6.7 supplemented with 5% fetal calf serum) then removal and replacement for 5 h with medium, 0.16 µM TPA, 135 µM AP or co-treatment (TPA + AP) respectively. For the fifth treatment AP 1 h + TPA 5 h = pre-treatment with 135 µM AP for 1 h then removal and replacement for 5 h with TPA. *Result significantly different from control ($P < 0.05$). Results are of triplicate determinations and a second experiment yielded similar results.

for each chemical (data not shown). Treatment of SHE cells for 5 h with 100 µM DEHP, 135 µM AP or 0.4 µM BaP alone did not change the rate of ODC activity, whereas the 5-h treatment with 0.16 µM TPA increased the ODC activity from 100% to ~250% (Figures 1, 2 and 3). Concomitant exposure of SHE cells to the drugs and TPA did not significantly modulate the effect of TPA on ODC activity. Surprisingly, 1 h pre-exposure to 100 µM DEHP or 0.4 µM BaP followed by 5 h exposure to 0.16 µM TPA treatment provoked a superstimulation of ODC activity: 71% and 35% respectively for DEHP and BaP as compared with TPA inducing rate. Using the same protocol with 135 µM AP did not give the same superinduction as above.

Effects of chemicals on SHE cell apoptosis

In SHE cell cultures a small rate of basal apoptosis regularly takes place, which may counterbalance cellular growth by mitosis. Treatment for 5 h with 4.6 µM camptothecin (CAM), an inhibitor of topoisomerase I, as well as fetal calf serum withdrawal, induced apoptosis and the rate of cell death increased by approximately twofold. Exposition of SHE cells for the same time to 100 µM DEHP, 135 µM AP or 0.4 µM BaP did not give any modification of basal DNA fragmentation (Table I). Using these chemicals in combination with serum withdrawal or CAM treatment did not significantly inhibit the induced apoptosis. On the contrary, using 0.16 µM TPA for 5 h slightly decreased basal SHE cells apoptosis by 20% (Table II). With sequential treatments, 1 h pre-exposure to DEHP, BaP and even to AP followed by 5 h of TPA exposure, greatly decreased the apoptotic rate and the inhibition was

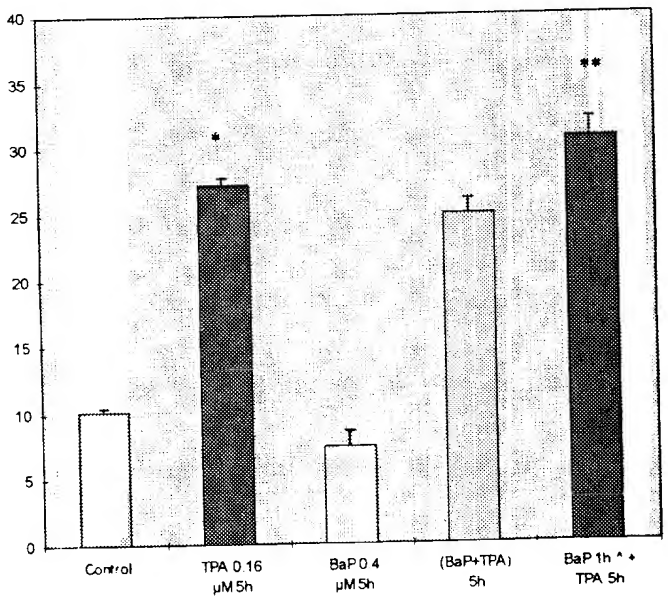

Fig. 3. Superinduction of ODC activity in SHE cells stage-exposed to benzo[*a*]pyrene and TPA. The four first treatments consisted of 1 h of treatment medium alone (DMEM medium pH 6.7 supplemented with 5% fetal calf serum) then removal and replacement for 5 h with medium alone, 0.16 µM TPA, 0.4 µM BaP or co-treatment (TPA + BaP) respectively. For the fifth treatment BaP 1 h + TPA 5 h = pre-treatment with 0.4 µM BaP for 1 h then removal and replacement with TPA for 5 h. *Result significantly different from control ($P < 0.05$); **ODC stimulation significantly different from that obtained with TPA alone ($P < 0.05$). Results are of triplicate determinations and a second experiment yielded similar results.

Table I. Effect of exposures to different chemicals alone on apoptosis of SHE cells

Treatments	Cellular fragmented DNA (AU)		
	w/10% FCS	w/o FCS	w/10% FCS w/CAM 4.6 μM
Control	100	212 ± 7*	254 ± 33*
DEHP 100 μM	105 ± 9	203 ± 12	219 ± 76
AP 135 μM	111 ± 55	200 ± 95	201 ± 43
BaP 0.4 μM	94 ± 7	203 ± 12	264 ± 29

Exposures to xenobiotics were performed either in DMEM pH 7.35 supplemented with 10% fetal calf serum (w/FCS) or in DMEM, pH 7.35, non-supplemented with FCS (w/o FCS) or in DMEM, pH 7.35, supplemented with 10% FCS and 4.6 μM camptothecin (w/FCS, w/CAM 4.6 μM).
*Significantly different from control w/10% FCS (<0.05). Result of the control w/FCS was set at 100 (arbitrary unit) and other results were expressed as compared with the control w/FCS. Results represent mean ± SD of quadruplicate determinations. A second independent experiment yielded similar results.

Table II. Inhibition of apoptosis in SHE cells staged-exposed to DEHP, AP, BaP and TPA

Treatments	DNA fragmentation (OD mean ± SD)	Fragmented DNA (AU)
Control	329.8 ± 18.8	100
TPA 0.16 μM 5 h	269.0 ± 13.0 *	82*
DEHP 1 h + TPA 5 h	208.3 ± 30.8 **	63**
AP 1 h + TPA 5 h	209.8 ± 15.0 **	64**
BaP 1 h + TPA 5 h	228.3 ± 18.3 **	69**

Treatment medium: DMEM medium, pH 7.35, supplemented with 10% fetal calf serum. The 5-h control and TPA treatments were preceded by 1 h in medium alone. Stage-exposure of SHE cells consisted of a 1-h treatment with 100 μM DEHP, 135 μM AP or 0.4 μM BaP then removal and replacement with 0.16 μM TPA for 5 h.
*Cellular DNA fragmentation significantly different from control ($P < 0.05$); **cellular DNA fragmentation significantly different from that obtained with TPA alone ($P < 0.05$). Results are of quadruplicate determinations and a second experiment yielded similar results.

20% more than that obtained with TPA alone. Since ODC superinduction was found to correlate with inhibition of apoptosis in SHE cells, we studied the effect of polyamines on apoptosis pattern. SHE cells were exposed to various doses of polyamines, namely putrescine, spermidine and spermine (10, 100 and 1000 μM). The compounds were diluted into the medium. After 5 h of exposure to polyamines, no inhibitory action could be obtained on SHE cells, even when the concentration of these agents increased to 1 mM (Table III).

Modification of Bcl-2 and Bcl-x_L proteins

Using the same exposure protocols, we studied the effects of carcinogens on the rate of two proteins known to inhibit apoptosis, Bcl-2 and Bcl-x_L. Bcl-2 oncoprotein increased by approximately threefold following 5-h of treatment with 0.16 μM TPA (Figure 4a). Sequential treatment with the carcinogen and TPA strongly increased this oncoprotein rate. With DEHP/TPA this proportion was ~12-fold greater than the control, and it was 8-fold greater with BaP/TPA. The increase was 6-fold in the presence of the non-carcinogenic compounds AP and TPA. Unlike Bcl-2, Bcl-x_L protein pathway appears not to be the target for carcinogen action. Stage-

Table III. Effect of exogenous polyamines (putrescine, spermidine and spermine) on spontaneous apoptosis of SHE cells

Concentration (μM)	Cellular fragmented DNA (AU)		
	Putrescine	Spermidine	Spermine
0	100 ± 6	100 ± 6	100 ± 6
10	93 ± 5	101 ± 4	93 ± 12
100	99 ± 4	90 ± 5	118 ± 12
1000	95 ± 1	91 ± 4	90 ± 8

Treatment medium: DMEM medium, pH 7.35, supplemented with 10% fetal calf serum. Result of the control was set at 100 (arbitrary unit) and other results were expressed as compared with the control. Results are of quadruplicate determinations and a second experiment yielded similar results.

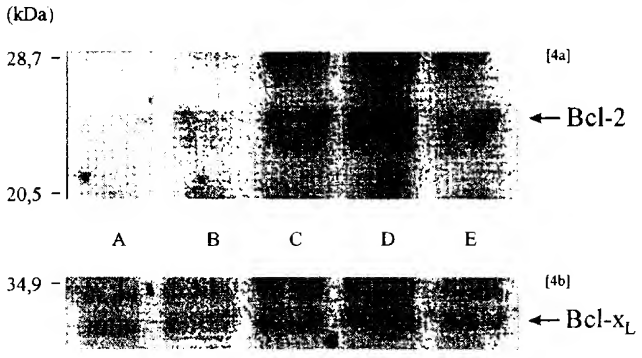

Fig. 4. Bcl-2 and Bcl-x_L immunoreactive proteins in SHE cells following two-stage exposures to xenobiotics (BaP, DEHP or AP) and phorbol ester (TPA). (A) control; (B) TPA 0.16 μM 5 h; (C) 1 h 0.4 μM BaP treatment then removal and replacement by TPA 0.16 μM for 5 h; (D) 1 h 100 μM DEHP treatment then removal and replacement by TPA 0.16 μM for 5 h; (E) 1 h 135 μM AP treatment then removal and replacement by TPA 0.16 μM for 5 h.

exposure of SHE cells to these chemicals did not change the Bcl-x_L rate as compared with the control (Figure 4b).

Discussion

Since the occurrence of apoptosis is a major event in cellular life, mechanistic studies of carcinogens have to take into account 'both sides of the growth equation' (43). The goal of this study was to explore what effects a non-genotoxic carcinogen (DEHP), a genotoxic carcinogen (BaP) and a non-carcinogen (AP) had on two opposite parameters that are often disturbed in the carcinogenic process, namely ODC and apoptosis.

The results reported in this study show that one-stage treatment of SHE cells with non-genotoxic carcinogen DEHP regularly decreased ODC basal level, whereas promoter carcinogen TPA strongly stimulated this ODC activity. However, pre-exposure of the cells to DEHP, even for a short time, potentialized the inducing effect of TPA on ODC activity. This ODC superinduction was obtained only with that two-stage exposure order: carcinogen–TPA. In contrast the same treatment protocol did not give any potentializing effect in the presence of the non-carcinogenic derivative AP. It is noteworthy that the same pattern of ODC superinduction could be obtained with the genotoxic carcinogen BaP provided that the compound was used in the initiation stage. In all cases reversing the stage exposure order to TPA–carcinogen, resulted in an inhibitory

pattern of TPA induced ODC. The mechanism of ODC superinduction in relation to stage-exposure of SHE cells to carcinogens remains to be clarified. Recent results have not shown any correlation between this superinduction and the ODC mRNA level. It is probable that post-transcriptional regulation of ODC could intervene in this mechanism and increase ODC protein and ODC activity. With regard to multistage SHE cell transformation assays, recent data have shown that repeated treatments with DEHP alone may increase the transformation frequency, moreover this was higher when DEHP was used at the initiation stage followed by promoter TPA (V.Cruciani and P.Vasseur, manuscript in preparation).

Our results clearly showed that the ODC superinduction in stage-exposed SHE cells to DEHP and TPA correlated with an inhibition of apoptotic rate in these cells. This was accompanied by increased levels of Bcl-2 oncoprotein, which is known to block the apoptotic process. Such apoptosis inhibition has been reported with non-genotoxic carcinogens (44). Nafenopin for example inhibited apoptosis in primary cultures of rat hepatocytes, in Reuber hepatoma cell line FaO (45) and *in vivo* in rat liver (46). The same inhibitory pattern was observed in this study with genotoxic carcinogen BaP in the two-stage treatment. The discrepancy appeared when non-carcinogenic derivative AP was used in the same stage-treatment: the compound inhibited apoptosis although it did not superinduce ODC activity. Although exposing SHE cells to the exogenous polyamines, putrescine, spermidine and spermine, did not exhibit any inhibitory effect on apoptosis, it is important to measure the uptake rate of polyamines by the cells. This may help to determine the respective role of the intracellular polyamines and the ODC signaling pathway in this apoptotic process. Since neoplasia was thought to be related to a disruption of equilibrium between cell proliferation and cell death resulting in a relative promotion of mutant cells, our results led to the assumption that a stage process of apoptosis could intervene in the mechanism of action of carcinogens. Two possible interaction pathways are that (i) decrease of apoptosis rate may be an early and prerequisite event in the first stage of cancer induction, and (ii) apoptosis inhibition may represent a primary cell reaction to various chemicals that is reflected by Bcl-2 protein accumulation. In the same stage-exposure to chemicals the other apoptosis inhibitor Bcl-x_L protein was not increased. It is probable that in relation to cell specificity, the Bcl-x_L pathway could not be the target for carcinogenic chemicals in SHE cell system. In any case, the two events, ODC superinduction and inhibition of apoptosis via Bcl-2 upregulation, seem to be essential for the development of carcinogenesis. One event only may not be sufficient with the non-carcinogenic compound AP.

In vivo experiments designed to assess carcinogenic potential of DEHP gave contradictory results, this could be due to some differences observed in experimental protocol. In 2-year studies where DEHP was fed to F344 rats and B6C3F1 mice, the compound significantly increased the incidences of hepatocellular carcinomas and adenomas in both sexes of rats and mice (4,47,48). The authors considered DEHP as a complete carcinogen, in that it acted both as initiator and promoter. In two-stage hepatocarcinogenesis studies DEHP did not exhibit any promoting action. Surprisingly, no initiating activity was found when DEHP was administered in the first stage followed by a promotion regimen (12). In two-stage rat renal tumorigenesis, DEHP was found to act as a tumor promoter and enhanced the carcinogenic action on renal tubules of *N*-ethyl-*N*-hydroxyethyl nitrosamine (49). Other authors could not find any promoting effect of DEHP on the urinary bladder of rats initiated first with *N*-butyl-*N*(4-hydroxybutyl) nitrosamine (50). The carcinogenesis mechanism of DEHP was extensively studied with several short-term *in vivo* assays. Identification of foci of cellular alteration in rat liver by multiple histochemical stains could not emphasize the promoting and the initiating activity of DEHP (12). In a 24-week assay based on hepatocellular altered foci or early neoplastic lesions, no evidence of carcinogenic potential of DEHP could be observed (13). It has been reported that DEHP treated rats developed positive altered foci in liver gammaglutamyl-transpeptidase (GGT). However these hepatic foci were morphologically and biochemically heterogeneous, also GGT was not regularly expressed in preneoplastic and neoplastic lesions of rats fed with peroxisome proliferators (48–51). The selection of the initiating agent may intervene in hepatocellular responses to PPs. Studies in which rats were initiated with diethylnitrosamine (DEN) and fed DEHP in the second stage, indicated that DEHP inhibited rather than promoted the formation of altered foci and carcinomas in liver (51). Comparative studies with a diet containing Clofibrate and WY-14643 showed that these PPs increased hepatoneoplastic rate following initiation with DEN and that they were inactive if 2-acetylaminofluorene was used at the initiation stage (52).

In a short-term assay using the SHE cell system, it has been reported that one-step treatment with DEHP (48 h) weakly increased morphological transformation frequency. This was enhanced when rat liver post-mitochondrial supernatant was added to the medium (53). In the standard SHE cell transformation assay, three applications of DEHP alone or DEHP followed by TPA increased the number of transformed colonies. In contrast, when SHE cells were pre-exposed to the initiator carcinogen BaP (0.40 μM) followed by DEHP at the promotion stage, the transformation frequency did not change. The results obtained with two-stage-exposure experiments, ODC superinduction and apoptosis inhibition through Bcl-2 upregulation, led to a high rate of transformed colonies and argues for DEHP action at the initiation stage of the carcinogenic process. However, in the classic interpretation of the combined action of the initiation–promotion protocol, the initiator is presumed to induce the formation of rare mutated or altered cells. Here, the contribution of DEHP appears not to be at the level of rare events, but at the level of behavior modification of the whole SHE cell population. That complete carcinogen can elicit not solely pure initiating effects is a widely acknowledged theory, and this seems to be valid for DEHP which cannot be considered a genotoxic carcinogen in the strict sense of the word.

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